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Inhibition of LPS-stimulated NO Production in Mouse Macrophage-like Cells by Benzocycloheptoxazines

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Abstract. *Twenty-six benzocycloheptoxazine derivatives were investigated for their effect on nitric oxide (NO) production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like RAW 264.7 cells. Benzo[b]cyclohepta[e][1,4]thiazine most effectively inhibited the LPS-stimulated NO production at noncytotoxic concentrations. 6H-Benzo[b]cyclohepta[e][1,4]-diazine cation, and benzo[b]cyclohepta[e][1,4]oxazine and its 6-bromo derivative also efficiently inhibited the LPS-stimulated NO production. Another sixteen benzo[b]cyclohepta[e]-[1,4]oxazine derivatives, 14H-[1,4]benzoxazino[3',2':3,4]-cyclohepta[1,2-b][1,4]benzoxazine and its 7-bromo- and 7-isopropyl derivatives were slightly less active (selectivity index (SI)=8.3-66). Bromination of benzo[b]cyclohepta[e][1,4]-thiazine, benzo[b]cyclohepta[e][1,4]oxazine and 2-methyl-benzo[b]cyclohepta[e][1,4]oxazine at C-6, C-8 or C-10 positions resulted in the significant reduction of the inhibitory activity. The observed inhibitory activity of benzo[b]cyclohepta-[e][1,4]thiazine and its 6,8-dibromo derivatives were not due to the reduction of the intracellular level of inducible NO synthase protein (based on Western blot analysis), nor to NO scavenging activity (based on ESR spectroscopy). These results suggest the possible anti-inflammatory action of benzocyclo-heptoxazines via inhibition of LPS-activated macrophages.*

Hinokitiol and its related compounds with a tropolone skeleton (1-3) have been reported to exhibit various biological activities such as antimicrobial (4), antifungal (5) and phytogrowth-inhibitory activity (6, 7), cytotoxic effects on mammalian tumor cells (8, 9) and inhibitory effects on catechol-*O*-methyltransferase (10) and metalloproteases (4). Hinokitiol acetate did not exhibit cytotoxic activity (9), antimicrobial activity nor metalloprotease inhibition (4), suggesting that these biological effects of hinokitiol-related compounds may result from the metal chelation of the carbonyl group at C-1 with the hydroxyl group at C-2 in the tropolone skeleton. Tropolones with a phenolic OH group, hinokitiol, its tosylate and methyl ethers have been found to exhibit higher tumor-specific cytotoxic activity, while 2-aminotropolone showed the highest tumor specificity and induced apoptosis in the human promyelocytic leukemia HL-60 cell line, possibly by radical-mediated redox reaction (11). 2,4-Dibromo-7-methoxytropone inhibited nitric oxide (NO) production by lipopolysaccharide (LPS)-stimulated mouse macrophage-like RAW 264.7 cells *via* the inhibition of inducible NO synthase (iNOS) expression, rather than *via* the radical-mediated mechanism (12).

Differing from unreactive heterocyclic-annulated tropylium compounds, cyclohepta[*b*][1,4]benzoxazines and their *S*- and *O*-analogues are usually very reactive, especially towards 1,4-difunctional nucleophiles such as *O*-phenylenediamine, ethylenediamine and their *S*- and *O*-analogues (13).

We have recently reported the cytotoxic activity of twenty-six benzocycloheptoxazines (Table I, Figure 1) against normal human oral cells (gingival fibroblast, pulp cell, periodontal ligament fibroblast) and human squamous cell carcinoma and promyelocytic leukemia HL-60 cell lines (14). Among eighteen benzo[*b*]cyclohepta[*e*][1,4]oxazine derivatives [**6-23**], 6,8,10-tribromo- [**9**], 6-bromo-2-methyl- [**20**] and 6-bromo-2-chloro- [**21**] derivatives showed the highest tumor-specific cytotoxicity. Compounds [**9, 20, 21**] induced apoptotic cell death in HL-60 cells, whereas they failed to induce apoptosis in HSC-4 cells (14). In order to explore these anti-inflammatory agents, we investigated here whether these twenty-six benzocycloheptoxazines (Table I, Figure 1) inhibit NO production by lipopolysaccharide (LPS)-stimulated RAW 264.7 cells.

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Table I. *Effect of benzocycloheptoxazines on NO production by LPS-stimulated RAW 264.7 cells.*

Compound		MW	CC_{50} (μM)	IC_{50} (μM)	SI
[**1**]	benzo[*b*]cyclohepta[*e*][1,4]thiazine	211	>500	1.08±0.19	>463.0
[**2**]	6,8-dibromobenzo[*b*]cyclohepta[*e*][1,4]thiazine	369	20.1±1.27	1.09±0.16	18.4
[**3**]	6,8,10-tribromobenzo[*b*]cyclohepta[*e*][1,4]thiazine	448	21.0±4.29	5.02±0.97	4.2
[**4**]	6*H*-benzo[*b*]cyclohepta[*e*][1,4]diazine	194	>10	0.45±0.07	>22.4
[**5**]	6*H*-benzo[*b*]cyclohepta[*e*][1,4]diazine cation	254	>50	0.58±0.08	>86.9
[**6**]	benzo[*b*]cyclohepta[*e*][1,4]oxazine	195	>100	1.14±0.28	>88.0
[**7**]	6-bromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	274	>100	1.15±0.17	>86.9
[**8**]	6,8-dibromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	353	>100	4.97±0.87	>20.1
[**9**]	6,8,10-tribromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	432	>10	0.72±0.10	>13.8
[**10**]	8-bromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	274	>100	5.92±0.32	>16.9
[**11**]	7-bromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	274	98.1±1.68	3.39±0.16	28.9
[**12**]	9-bromobenzo[*b*]cyclohepta[*e*][1,4]oxazine	274	46.1±6.81	1.72±0.07	26.8
[**13**]	8-isopropylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	236	91.7±11.7	3.33±0.42	27.6
[**14**]	9-isopropylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	236	>100	4.77±0.63	>20.9
[**15**]	6-acetoxybenzo[*b*]cyclohepta[*e*][1,4]oxazine	253	96.2±6.64	4.15±0.68	23.2
[**16**]	8-acetoxybenzo[*b*]cyclohepta[*e*][1,4]oxazine	253	>10	0.62±0.07	>16.3
[**17**]	8-bromo-6-acetoxybenzo[*b*]cyclohepta[*e*][1,4]oxazine	332	>100	6.19±1.18	>16.2
[**18**]	9-acetoxybenzo[*b*]cyclohepta[*e*][1,4]oxazine	253	>100	1.51±0.09	>66.2
[**19**]	2-methylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	209	95.9±7.16	4.08±0.27	23.5
[**20**]	6-bromo-2-methylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	288	78.9±12.4	9.46±1.36	8.3
[**21**]	6-bromo-2-chlorobenzo[*b*]cyclohepta[*e*][1,4]oxazine	308	>10	1.39±0.06	>7.2
[**22**]	8-bromo-2-methylbenzo[*b*]cyclohepta[*e*][1,4]oxazine	288	>100	4.93±0.58	>22.8
[**23**]	8-bromo-2-chlorobenzo[*b*]cyclohepta[*e*][1,4]oxazine	308	>100	12.0±0.20	>9.1
[**24**]	14*H*-[1,4]benzoxazino[3',2':3,4]cyclohepta[1,2-*b*][1,4]benzoxazine	300	>100	1.50±0.11	>66.7
[**25**]	14*H*-7-bromo[1,4]benzoxazino[3',2':3,4]cyclohepta[1,2-*b*][1,4]benzoxazine	379	>10	0.35±0.05	>28.7
[**26**]	14H-7-isopropyl[1,4]benzoxazino[3',2':3,4]cyclohepta[1,2-*b*][1,4]benzoxazine	342	>10	0.19±0.02	>52.9

MW: Molecular weight; CC_{50}: 50% cytotoxic concentration; IC_{50}: concentration at which NO production was inhibited by 50%; SI: Selectivity index (CC_{50}/IC_{50}). Each value represents the mean from 3 independent experiments.

Materials and Methods

Materials. The following chemicals and reagents were obtained from the companies indicated: Dulbecco's modified Eagle's medium (DMEM), phenol red-free DMEM (Gibco BRL, Grand Island, NY, USA); fetal bovine serum (FBS) (JRH Bioscience, Lenexa, KS, USA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), LPS from *Escherichia coli* (Serotype 0111:B4) (Sigma Chem. Co., St. Louis, MO, USA); 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxy1-3-oxide (carboxy-PTIO; a spin trap agent), 1-hydroxy-2-oxo-3-(*N*-3-methyl-3-aminopropyl)-3-methyl-1-triazene (NOC-7; an NO generator) (Dojin, Kumamoto, Japan); dimethyl sulfoxide (DMSO) (Wako Pure Chem. Ind., Ltd., Osaka, Japan).

Synthesis of benzocycloheptoxazines. Benzocycloheptoxazine derivatives (Table I, Figure 1) were synthesized according to the methods previously reported (14).

Cell culture. RAW 264.7 cells were cultured in DMEM supplemented with 10% heat-inactivated FBS under a humidified 5% CO_2 atmosphere.

Assay for NO production. RAW 264.7 cells were initially inoculated at 6×10^4/0.1 ml in 96-microwell plates (Becton Dickenson, Labware, NJ, USA). Media were replaced with fresh phenol red-free medium supplemented with 10% FBS and different concentrations (0-100 or 500 μM) of test samples in the absence or presence of LPS (0.1 μg/ml), and incubated for a further 24 hours. The relative viable cell number was then measured by the MTT method (12, 14, 15). The 50% cytotoxic concentration (CC_{50}) was determined from the dose–response curve. The NO produced in the culture medium was quantified by Greiss reagent, using a standard curve of NO_2 (12). The concentration at which NO production was inhibited by 50% (IC_{50}) was determined from the dose–response curve. The extent of the inhibition of NO production was monitored by the selectivity index (SI) defined as follows: $SI=CC_{50} / IC_{50}$.

Western blotting. The pelleted cells were lysed with 100 μl of lysis buffer (10 mM Tris-HCl, pH 7.6, 1% Triton® X-100, 150 mM NaCl, 5 mM EDTA and 2 mM phenylmethylsulfonyl fluoride) for 10 minutes in iced water. The cell lysates were centrifuged at 16,000×g for 20 minutes at 4˚C to remove insoluble materials and the supernatant was collected. The protein concentrations of supernatants were measured by Protein Assay Kit (Bio Rad, Hercules, CA, USA). An equal amount of the protein from cell lysates (10 μg) was mixed with 2x sodium dodecyl sulfate (SDS)-sample buffer [0.1 M Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.01% bromophenol blue, 1.2% 2-mercaptoethanol], boiled for 10 minutes, applied to SDS-8% polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% skimmed milk in phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(–)] plus 0.05% Tween 20 for 90

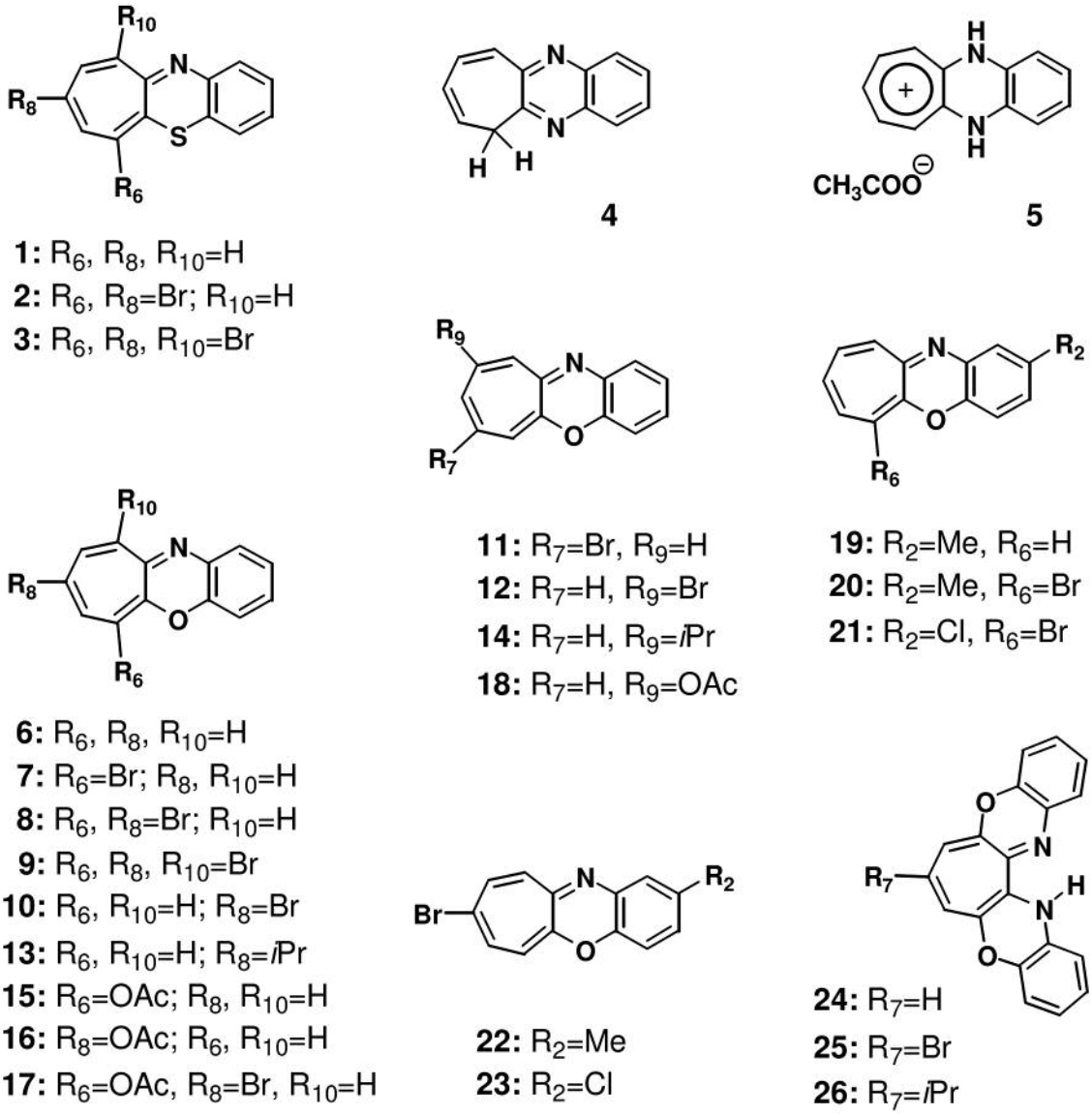

Figure 1. *Structures of twenty-six benzocycloheptoxazines used in this study.*

minutes and incubated for 90 minutes at room temperature with anti-iNOS antibody (1:1,000; Santa Cruz Biotechnology, Delaware, CA, USA), or anti-actin antibody (1:2000-4000; Sigma), and then incubated with horseradish peroxidase-conjugated anti-rabbit or mouse IgG for 90 minutes at room temperature. Immunoblots were developed with a Western Lightning™ Chemiluminescence Reagent Plus (Perkin Elmer Life Sciences, Boston, MA, USA) (15).

NO radical scavenging activity. The radical intensity of benzocycloheptoxazines was determined at 25˚C, using electron spin resonance (ESR) spectroscopy (JEOL JES REIX Tokyo, Japan, X-band, 100 kHz modulation frequency) (15). The radical intensity of NO, produced from the reaction mixture of 20 μM carboxy-PTIO and 60 μM NOC-7, was determined in 0.1 M phosphate buffer, pH 7.4 in the presence of 30% DMSO (microwave power and gain were changed to 5 mW and 400, respectively). When NOC-7 and carboxy-PTIO were mixed, NO was oxidized to NO_2 and carboxy-PTIO was reduced to carboxy-PTI, which produces seven-line signals. Samples were added 3 minutes after mixing. The NO radical intensity was defined as the ratio of the signal intensity of the first peak of carboxy-PTI to that of MnO an external marker, and expressed as the ratio to the height of MnO. The concentration that reduced the NO radical intensity by 50% (EC_{50}) was determined from the dose-response curve.

Results

Inhibition of NO production. LPS (0.1 μg/ml) significantly stimulated NO production by RAW 264.7 cells, and the NO released into the culture medium was elevated from the background level to approximately 39 μM (data not shown). Benzocycloheptoxazine alone did not significantly induce NO

production by unstimulated RAW 264.7 cells (data not shown), but effectively inhibited the NO production by LPS-stimulated RAW 264.7 cells. Among twenty-six benzocyclo-heptoxazine derivatives, benzo[*b*]cyclohepta[*e*][1,4]thiazine [**1**] potently inhibited the NO production by the activated RAW 264.7 cells (IC_{50}=1.08 μM), and its cytotoxicity was detected only at two orders higher concentrations (CC_{50}>500 μM) (Figure 2A), yielding the highest selectivity index (SI>463.0) (Table I). Although 14*H*-7-isopropyl[1,4] benzoxazino[3',2':3,4] cyclohepta[1,2-*b*][1,4]benzoxazine [**26**] has the lowest IC_{50} and therefore is most potent at NO inhibition, it is more cytotoxic (CC_{50}>10 μM) than [**1**], yielding a lower selectivity index (SI>52.9) (Table I). Compounds [**2, 3**], that is 6,8-dibromo and 6,8,10-tribromo derivatives of [**1**], respectively, exhibited higher cytotoxicity (CC_{50}=20-21 μM), and therefore much lower SI values (SI=18.4 and 4.2, respectively) (Figure 2B, Table I). 6*H*-Benzo[*b*]cyclohepta[*e*][1,4]diazine cation [**5**], and benzo[*b*] cyclohepta[*e*][1,4]oxazine [**6**] and its 6-bromo derivative [**7**] also exhibited higher SI values. Another sixteen benzo[*b*] cyclohepta[*e*][1,4]oxazines [**8-23**] and 14*H*-[1,4]benzoxazino [3',2':3,4]cyclohepta[1,2-*b*][1,4]benzoxazine [**24**] and its 7-bromo-[**25**] and 7-isopropyl- [**26**] derivatives also inhibited LPS-stimulated NO production to slightly lower extents (SI=8.3-66).

Effect on iNOS level. Compounds [**1, 2**] did not reduce the intracellular level of inducible NO synthase, as shown by Western blot analysis (Figure 3A, 3B).

NO scavenging activity. ESR spectroscopy showed that compound [**1**] dose-dependently scavenged the NO radical produced by NOC-7, with an EC_{50} of 485 μM (Figure 4A). The NO scavenging activity of [**2**] was much less (EC_{50}>1219 μM) (Figure 4B).

Discussion

Benzo[*b*]cyclohepta[*e*][1,4]thiazine [**1**] effectively inhibited the NO production by LPS-stimulated RAW 264.7 cells (SI>463), without showing any apparent cytotoxicity (CC_{50}>500 μM). The introduction of two or three bromines at the C-6 and -8 positions, or at the C-6, -8, and -10 positions of the cycloheptane ring [**2, 3**] significantly enhanced the cytotoxicity and therefore showed much lower inhibition of NO production, as revealed by the reduction of the SI values (18.4 and 4.2, respectively). This finding is consistent with our previous report that bromination of benzo[*b*]cyclohepta[*e*][1,4]thiazine [**1**] resulted in significant enhancement of its cytotoxicity against both normal and tumorous human cells (14).

Similarly, the inhibitory effect of benzo[b]cyclohepta [e][1,4]oxazine [**6**] on NO production by LPS-activated RAW 264.7 cells (SI>88.0) was reduced by bromination at the C-6 and -8 positions [**8**] (SI>20.1), and at the C-6, -8, and -10 positions [**9**] (SI>13.8). The inhibitory effect of 2-methylbenzo[*b*]cyclohepta[*e*][1,4]oxazine [**19**] (SI=23.5) was also reduced by bromination at the C-6 position [**20**] (SI=8.3) (Table I). These data suggest negative correlation between the cytotoxicity of benzocycloheptoxazines and the inhibitory activity against NO production by activated macrophages.

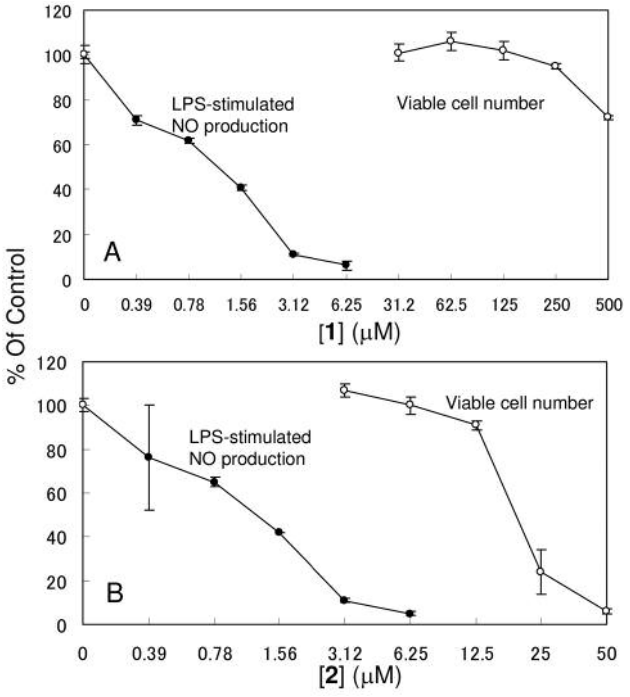

Figure 2. *Inhibition of NO production by LPS-stimulated RAW 264.7 cells by benzocycloheptoxazines. Near confluent RAW 264.7 cells were incubated for 24 hours without (control), or with the indicated concentrations of benzo[b]cyclohepta[e][1,4]thiazine [**1**] (A) or its 6,8-dibromo derivative [**2**] (B) in the presence of 0.1 μg/mL LPS in phenol red-free DMEM supplemented with 10% FBS. The viable cell number (○) and extracellular concentration of NO (●) were then determined by MTT assay and Griess reagent, respectively. Each value (expressed as % of control) represents mean±S.D. of triplicate assays.*

We have previously reported that compounds [**9, 20, 21**] showed the highest tumor-specific cytotoxicity (14). The present study, however, demonstrated that these compounds inhibited the LPS-stimulated NO production to a much lesser extent (SI=>13.8, 8.3, >7.2, respectively) (Table I). This suggests the lack of a correlation between the tumor specificity and the inhibitory activity against the NO production by activated macrophages.

There are two possibilities that explain the decline of the NO production by activated macrophages: either NO production is inhibited or NO degradation is increased. ESR demonstrated that the second possiblity can be rejected, since the NO scavenging activity of [**1, 2**] was very weak. Therefore, the first possibility should be considered. So far, we found that compounds [**1, 2**] failed to reduce the intracellular concentration of iNOS. The

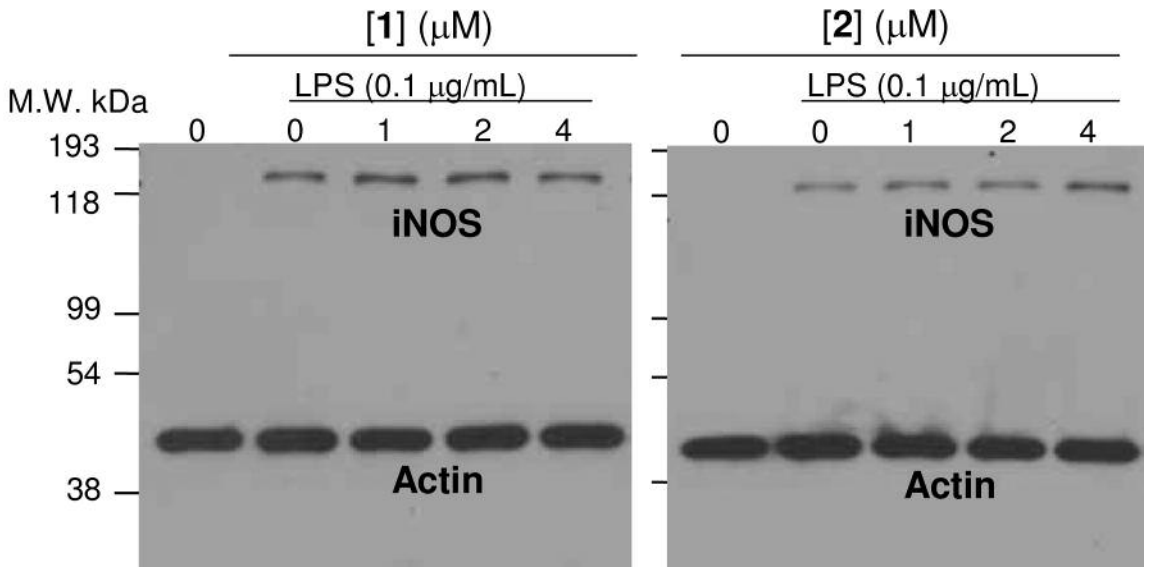

Figure 3. *Effect of benzocycloheptoxazine derivatives [**1**, **2**] on the intracellular level of iNOS protein. RAW 264.7 cells were incubated for 24 hours with or without 0.1 μg/mL LPS with the indicated concentrations of [**1**] or [**2**], and the iNOS protein expression was monitored by Western blot analysis. Representative data from one of two independent experiments are shown.*

possibility that these compounds may inactivate iNOS activity remains to be investigated. Whatever the underlying mechanism, the present study suggests the possible anti-inflammatory action of benzocyclo-heptoxazines, based on their inhibition of LPS action against macrophages. To test whether benzocyclo-heptoxazines actually has any anti-inflammatory action, the effects on prostaglandin production and cyclooxygenase expression in the activated macrophages should be investigated.

Acknowledgements

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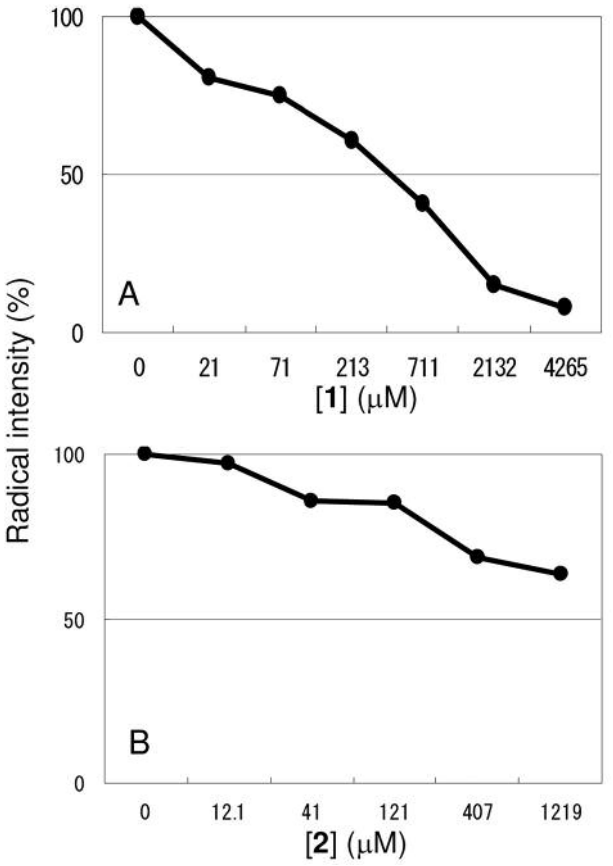

Figure 4. *NO radical scavenging activity of benzocycloheptoxazine derivatives [**1**, **2**]. Representative data from one of two independent experiments are shown.*

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